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Abscisic Acid During Zygotic Embryogenesis in *Pinus Taeda L.*

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ABSCISIC ACID DURING ZYGOTIC EMBRYOGENESIS IN *PINUS TAEDA* L.

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Abstract. -- Indirect ELISA was used to quantify abscisic acid (ABA) in developing zygotic embryos and megagametophytes from two loblolly pine (*Pinus taeda* L.) mother trees. Similar trends in (+)-ABA concentrations were found in tissues taken from either mother trees. On a dry-weight basis, embryonic and megagametophytic ABA levels were highest in early development and decreased steadily to their lowest levels at cone ripening. Embryonic ABA was significantly higher than megagametophytic ABA. On an organ-basis, embryonic ABA was lowest during early to mid-development; it increased rapidly during mid-development, then declined and remained constant (UC) or dropped off quickly (WV) at cone ripening (late development). Total ABA per embryo increased rapidly prior to the rapid increase in dry-weight accumulation in the embryos of both mother trees. On the other hand, total per megagametophyte remained fairly constant through all stages of development, with the exception of mid-development, where it dropped, then increased significantly before leveling off and remaining constant.

Keywords. -- ABA, abscisic acid, zygotic embryogenesis, conifer, loblolly pine, seed maturation, ELISA, enzyme-linked immunosorbant assay, somatic embryogenesis.

INTRODUCTION

Endogenous abscisic acid (ABA) has long been considered a major factor in proper seed development and maturation. Putative roles for ABA in seed development include inhibition of precocious germination, promotion of storage protein synthesis and desiccation tolerance, suppression of reserve mobilization, and induction of dormancy (Black 1991). During somatic embryogenesis in *Picea glauca* (Attree et al. 1992), *Picea glauca engelmanni* (Roberts 1991), *Picea abies* (Hakman et al. 1990), and *Pinus taeda* (Becwar and Feirer 1989), exogenous ABA appears to serve similar critical functions. Specifically, ABA

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inhibits precocious germination, and stimulates the accumulation of storage nutrients, lipids, and proteins in embryos developed in culture. ABA, combined with polyethylene glycol, improved desiccation tolerance in somatic embryos of *Picea glauca* by enhancing lipid biosynthesis (Attree et al. 1992).

Very little is known about changes in ABA levels or, for that matter, many of the other biochemical and molecular events that occur during conifer zygotic embryogenesis. In fact, there is no published literature pertaining to changes in ABA during zygotic development in gymnosperms. However, from what evidence exists, ABA appears to play a critical role in the proper development of zygotic and somatic conifer embryos.

The objective of our research was to gain a better understanding of the role played by ABA in determining morphological events during zygotic embryogenesis in Loblolly pine. This was accomplished by staging zygotic embryos based on their morphological features (i.e., appearance of apical meristem, cotyledons, etc.), and then measuring the ABA levels in embryos and megagametophytes from fertilization to cone ripening (June-October) using an indirect enzyme-linked immunosorbant assay (ELISA). Results from this study will be used to develop a model to assist in determination of optimal ABA requirements for a somatic embryogenesis system.

MATERIAL AND METHODS

ELISA/Chemicals and Supplies. *Buffers.* Phosphate-buffered saline plus Tween (PBS/Tween): 13.6 g potassium monophosphate, 29.2 g sodium chloride per liter containing 0.05% Tween 20 (pH 7.4); blocking solution: 5% (w/v) bovine serum albumin (BSA) (Sigma, A-3425) in PBS/Tween; assay buffer: 0.1% (v/v) BSA in PBS/Tween. *ABA-4'-BSA Conjugate.* The conjugate was prepared according to Quarrie and Galfre (1985) and stored at -20°C in its concentrated form. The conjugate was diluted in 0.05 M sodium carbonate (pH 9.6) to a concentration (1:100,000) as determined by optimization trials. *Monoclonal Antibody.* The monoclonal antibody (mAb) raised against free *cis*-(+)-ABA (Mertens et al. 1983) was from Idetek, Inc., (San Bruno, CA). The mAb was dissolved in 2 mL assay buffer, aliquoted into 10 µL portions, and stored at -80°C. The monoclonal was further diluted in assay buffer to a final concentration (1:5,000) as determined by optimization trials. *Goat Antimouse Antibody-Biotin Conjugate.* Goat antimouse antibody-biotin conjugate (Sigma, B-7264) was aliquoted into 10 µL portions and stored at -20°C. The conjugate was diluted 1:5,000 in assay buffer prior to use. *Streptavidin-Poly-HRP Conjugate.* Streptavidin-Poly-HRP (Research Diagnostics, Inc., Flanders, NJ, No. RDI-pHRP20-SA) was aliquoted into 10 µL portions and stored at -20°C. The conjugate was diluted 1:5,000 in assay buffer prior to use. *Tetramethyl-benzidine (TMB) Substrate.* A 10 mg/mL solution of TMB (Sigma, T-2885) in Dimethyl sulfoxide was diluted 1:1,000 in 100 mM sodium acetate (pH 5.5). Hydrogen peroxide (3% solution) was added to a final concentration of 0.002%. *Abscisic Acid (ABA) Standards.* A

stock solution of 50 mM (+)-*cis*-ABA was prepared by dissolving (±)-*cis,trans*-ABA (Sigma, A-1012) in absolute methanol. This solution was stable for at least three months when stored at -20°C in the dark. Standards ranging from 15.8 ng to 5 pg/100 µL (+)-ABA were made in assay buffer by serial dilution. The small amount of methanol in the standards did not affect the performance of the antibody (data not presented). Assay range was 2.5 ng to 2.5 pg/100 µL (+)-ABA. *Microtiterplates.* Immulon-2, flat-bottom, 96-well microtiter plates (Dynatech Laboratories, Chantilly, VA) were found to yield optimal binding of the ABA-4'-BSA conjugate (Walker-Simmons 1987).

Plant Material. Loblolly pine (*Pinus taeda* L.) cones from two seed orchards were shipped overnight, on ice packs, on a weekly basis from June to October 1992 (fertilization to cone ripening). The two mother trees were designated as UC (Bellville, GA) and WV (Summerville, SC). Seeds were extracted from the cones immediately upon delivery, and embryos, suspensors, and megagametophytes were separated under a stereoscopic microscope in the cold. The embryos were staged for morphological development and briefly rinsed in distilled water to remove extraneous hormones that may have leaked from the megagametophyte during dissection. The three tissues were frozen separately in liquid nitrogen, lyophilized, and stored at -80°C until analyzed. Tissues were stored based on mother tree, tissue type, and stage of development.

Staging of Developing Embryos. Staging of the tissues was based on morphological features of the developing embryos using a method developed at Institute of Paper Science and Technology (Webb 1991). Stage 1: proembryos, from free nucleate to 12 cells, approximately 3 days since fertilization (DSF); Stage 2: embryo proper, microscopic but distinct/translucent, still found at the micropylar end of megagametophyte, 6 DSF; Stage 3: embryo proper becomes white/opaque and is found at chalazal end of megagametophyte, 39 DSF; Stage 4: larger than Stage 3, opaque embryo proper enlarged longitudinally, dome-shaped apex, 43 DSF; Stage 5: similar to Stage 4, except apical meristem is visible, 45 DSF; Stage 6: similar to Stage 5, except cotyledon primordia are barely visible below apical meristem, 47 DSF; Stage 7: similar to Stage 6, except cotyledons are elongated, but do not overtop apical meristem, 49 DSF; Stage 8A: similar to Stage 7, except cotyledons overtop apical meristem, apical meristem still visible, 52 DSF; Stage 8B: similar to Stage 8A, except embryo and cotyledon lengths are longer, 57 DSF; Stage 9: cotyledons curved and joined at their tips; apical meristem is not visible from any angle. Stage 9 tissue was collected weekly and subdivided by percent moisture content of tissue since growth continued without obvious morphological change (9A-9H).

ABA Analysis. ABA Extraction. Isolated tissues were ground using a cold glass rod, weighed, placed in screw-cap teflon tubes, and extracted in 10 mL 80% methanol (v/v) containing 25 mg/L butylated hydroxytoluene (BHT) adjusted to pH 7 (Neill and Horgan 1987, Niell et al 1983, Milborrow and Mallaby 1975). Approximately $5x10^5$ DPM of [^{3}H]-ABA (Amersham, Arlington Heights, IL, TRK.644) was added as an internal standard. The tubes were purged with nitrogen gas, and the

tissues were extracted overnight with stirring at 4°C in the dark.

ABA Purification. The homogenate was centrifuged at 2000x*g* for 15 minutes, and the supernatant was removed. The pellet was resuspended in 1 mL of extracting solvent and recentrifuged at 2000x*g* for 10 minutes. The supernatants were pooled, passed through a 0.45 µm nylon filter, and reduced to near dryness using rotoevaporation *in vacuo* at 35°C and dim lighting. The remaining aqueous phase was diluted to 1000 µL in assay buffer for analysis by indirect ELISA. This procedure averaged an ABA recovery of 81% (95% CI 3%).

ABA ELISA Quantification. This procedure was modified from Walker-Simmons (1987) by substitution of a biotin-streptavidin-multiple horseradish peroxidase (HRP) system for added amplification. *Coating of microtiter plate.* Diluted ABA-4'-BSA conjugate (200 µl) was added to each well of the microtiter plate except those in the outside rows as they were previously shown to produce inconsistent results (Ross et al. 1987). The plates were sealed with Parafilm and incubated overnight at 4°C in the dark. *Blocking of the wells.* Plates were aspirated and washed four times with PBS/Tween. Approximately 300 µL of blocking solution were added to each well, and left to incubate for 45 minutes at 37°C in the dark. The wells were aspirated and washed four times with PBS/Tween. *Incubation of standards or samples with ABA antibody.* ABA standards and samples were diluted 1:1 with the diluted monoclonal antibody and incubated overnight at 4°C in the dark. Samples were diluted in assay buffer to obtain absorbencies in the center of the calibration curve. Aliquotes (200 µl) from the samples or standards incubated with monoclonal antibody were added to the plate, which was then incubated for 90 minutes at 37°C in the dark. The wells were aspirated and washed four times with PBS/Tween. *Addition of goat antimouse antibody-biotin conjugate.* Diluted secondary antibody-biotin conjugate (200 µl) was added to each well, and the plate was incubated for 90 minutes at 37°C in the dark. The wells were aspirated and washed four times with PBS/Tween. *Addition of Streptavidin-Poly-HRP conjugate.* Diluted streptavidin-Poly-HRP conjugate (200 µl) was added to each well, and the plate was incubated for 90 minutes at 37°C in the dark. The wells were aspirated, washed four times with PBS/Tween, and once with 300 µL/well of 100 mM sodium acetate to remove potassium salt. *TMB reaction.* TMB solution (200 µl) was added to each well, and the blue color was allowed to develop at room temperature in the dark until a B_0 absorbance of approximately 0.40 at 630 nm using a Bio-Tek microplate reader. Reactions were stopped with 40 µl of 1.5 *N* H_2SO_4 and the absorbance was read at 450 nm. The concentration of ABA in the sample was inversely proportional to the absorbance. Triplicate ABA standards were assayed on each plate. A logit transformation was performed on the absorbencies of the standards to linearize the calibration curve (Weiler 1986). The quantity of (+)-ABA in the samples was calculated by performing the logit transformation and comparing the results to the calibration curve of (+)-ABA for each plate.

GC/MS-SIM Validation of the Indirect ELISA. Samples extracted for validation of the indirect ELISA were purified as stated above and taken to complete dryness using rotoevaporation *in vacuo* at 37°C and dim lighting. Samples were methylated with ethereal diazomethane, and ABA levels were quantified by GC/MS-SIM using $^2H_6$-ABA as an internal standard (Neill and Horgan 1987). The analysis was performed on a Hewlett-Packard 5890 GC with direct capillary interface to a Hewlett-Packard 5971A mass-selective detector. The GC program was 60°C for 2 min., 25°C/min. to 165°C, then 5°C/min. to 275°C. The helium gas flow was 0.5 mL/min. The capillary column was a 15 m x 0.25 mm x 0.25 µm film DB1 (J&W Scientific, Ltd.). The injection and interface temperatures were 300°C. Data were collected using the SIM program, monitoring four ions: m/e 190 (endogenous ABA), m/e 194 ($^2H_6$-ABA), and m/e 162 and 166 to monitor impurities. ELISA validation by GC/MS-SIM using late stage tissue demonstrated good agreement between the two methods and showed no cross-reactants or interferents to the ELISA (data not presented).

Statistical Analysis. Statistical significance of the multiple pairwise comparisons for ABA in the tissues was based on Bonferroni-Welch which assumes normal distributions but unequal variances between samples (Neter et al. 1985, Welch 1938). Analysis was carried out at 95% confidence intervals (α=0.05).

RESULTS AND DISCUSSION

ABA Levels in Developing Tissues

Changes in ABA levels found in developing embryos and megagametophytes are illustrated for seeds taken from the UC and WV trees on a per-organ basis (Figure 1) as well as on a dry-weight basis (Figure 2). Each data point represents an average of one to four extractions, depending on the amount of available tissue, with 11 assay replicates per extraction (11 to 44 samples per data point). The error bars represent the 95% confidence intervals. Spacing of the data points along the abscissa represents stages of embryo development as they occur in nature, i.e., rapid morphological development in Stages 4-8A is followed by slower development during maturation. ABA was detected in all tissues tested.

Measurements of ABA levels during zygotic embryogenesis in gymnosperms have not been previously published. Therefore, changes in ABA levels in Loblolly pine were compared with trends in herbaceous species. In many herbaceous species, ABA has been found to be extremely low or undetectable during early development. ABA levels tend to increase to maximal levels by the midpoint of the maturation process, and subsequently decrease to low levels by full maturity (Groot et al 1991, Xu et al. 1990, Napier et al. 1989, Quarrie et al. 1988). This trend was not found on a per-organ basis in developing embryos from either of the loblolly mother trees (Figure 1). In pine embryos, there was a significant increase in ABA from Stages 8A to 9A, followed by either a decline and a second increase for the UC mother tree, or a

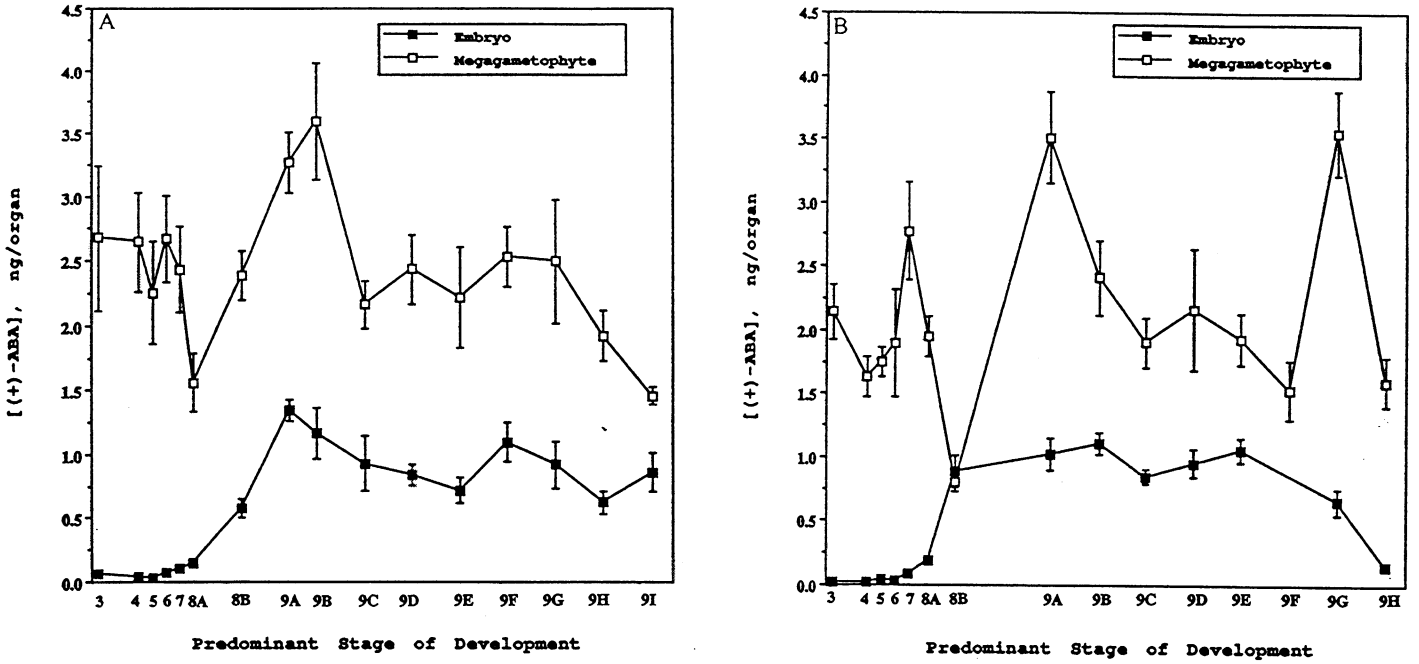

Figure 1. ABA levels in developing embryos and megagametophytes from (**A**) UC and (**B**) WV from Stage 3 to cone ripening (June-October 1992), on a per-organ basis. Stage 9 tissue was collected weekly and divided into additional substages A-H. Error bars represent the 95% confidence intervals.

rapid drop for the WV tree. This trend was similar to that found for ABA levels in embryos from *Acer platanoides* L. (Pinfield and Gwarazimba 1992), *Prunus persica* L. cv Springcrest (Piaggesi et al. 1991), *Phaseolus coccineus* (Perata et al. 1990), and *Pisum sativum* (Wang et al. 1987).

It has also been suggested that embryos synthesize their own ABA (Bray and Beachy 1985). However, as shown in Figure 1 for Loblolly pine embryos, the first increase in ABA occurred concomitantly with an apparent decrease in ABA levels in the megagametophyte. This suggests that these embryos may "import" ABA from the megagametophyte. Given that no direct vascular connection exists between the developing embryo and the rest of the plant, it is possible that the embryo takes all of its water and nutrients from the megagametophyte tissues (Morris et al. 1991). Rates of transfer for water and nutrients are likely to be greatest during early tissue development when moisture levels are high and the tissues are in intimate contact. During later stages of development, when desiccation is rapid, the outer cell layers of the embryo and megagametophyte dry out and break close contact, thus restricting the movement of water and nutrients. This might explain this rise in megagametophytic ABA without a corresponding change in embryonic ABA noted in the WV tree at Stage 9F.

Megagametophytic ABA levels at stages 8A and 9A/9F were signifi-

cantly discrepant (lower and higher, respectively) from levels found at all other stages; however, no other significant changes in megagametophytic ABA levels were detected. Megagametophytic ABA levels were significantly higher than in the corresponding embryo tissue, a trend that appears to be species-dependent (Piaggesi et al. 1991, Jones and Brenner 1987).

Generalizations from developing herbaceous embryos were not applicable to ABA levels as measured in Loblolly pine embryos on a per g dry-weight basis (Figure 2). Overall, the highest embryonic ABA levels were found at early development, followed by a steady decline to their lowest levels at cone ripening. This trend has been observed in embryos of *Zea mays* L. (Jones and Brenner 1987) and *Triticum aesitivum* L. (Walker-Simmons 1987). There appeared to be at least two significant peaks in Loblolly pine embryos; these occurred at Stages 7 and 9A for the UC mother tree, and Stages 3 and 8A for the WV mother tree. Two peaks for ABA have been reported in developing embryos of *Phaseolus coccineus* (Perata et al. 1990) and *P. vulgaris L.* (Hsu 1979), and three to four peaks have been observed in *Brassica napus L.* (Finkelstein et al. 1985). ABA peaks in these plants did not necessarily occur at the same stage of embryo development. Embryonic ABA in Loblolly pine was significantly higher than that in the megagametophyte tissue. Again, this trend appears to be species-dependent (Xu et al. 1990, Quarrie et al. 1988, Jones and Brenner 1987).

Levels and Dry-weight Accumulation in Developing Embryos

Changes in embryonic ABA relative to dry weight are displayed in Figure 3. A rapid increase in ABA occurred just prior to an increase in dry weight in embryo from either mother tree. This trend has been observed in many species, including *Zea* (Jones and Brenner 1987), *Glycine max.* (Ackerson 1984), and *Triticum aestivum* L. (King 1976). These late stage peaks in Loblolly pine may be involved in the production of proteins which can confer desiccation tolerance to the mature embryos. Although increases in ABA appear attributable to increases in Loblolly pine embryo dry mass, further evidence is necessary to substantiate such a claim. It has been observed in *Gossypium* that high levels of specific proteins (known as *Lea* for Late Embryogenesis Abundant) coincide with the rise in endogenous seed ABA levels, and that these proteins can also be prematurely induced by incubation of immature seeds in ABA (Skriver and Mundy 1990, Galau et al. 1986).

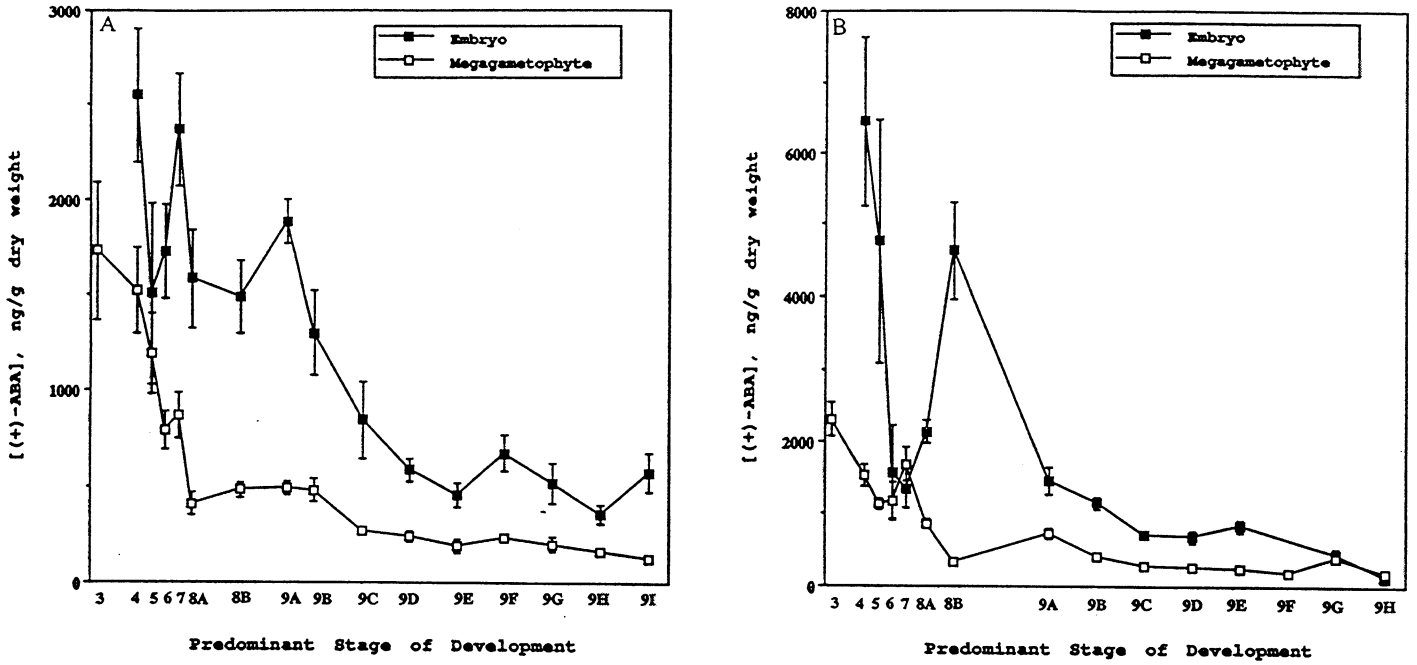

Figure 2. ABA levels in developing embryos and megagametophyte from (**A**) UC and (**B**) WV from Stage 3 to cone ripening (June-October 1992), on a per g dry-weight basis. Stage 9 tissue was collected weekly and divided into additional substages A-H. Error bars represent the 95% confidence intervals.

CONCLUSIONS

Thus far in our work, similar trends in (+)-ABA levels were found in seed tissues from two Loblolly pine mother trees grown in different locales, both on a g dry-weight was well as on an organ basis. The use of mother trees (half-sibs or open pollinated) grown in two different seed orchards is important in developing observations to be extended to all Loblolly pines. Fertilizations were performed by multiple father trees, and thus, trends in seed ABA level fluctuations are likely to be common to most developing Loblolly pine seeds.

ABA peaks did not appear to correspond to any observable morphogenic change in the embryos. However, when measured on a per-embryo basis, a rapid increase in ABA did occur immediately prior to an increase in dry weight. Although a causal relationship is suggested, it is not known if the increase in ABA levels is the cause of the increase in embryo dry mass (late stage protein synthesis).

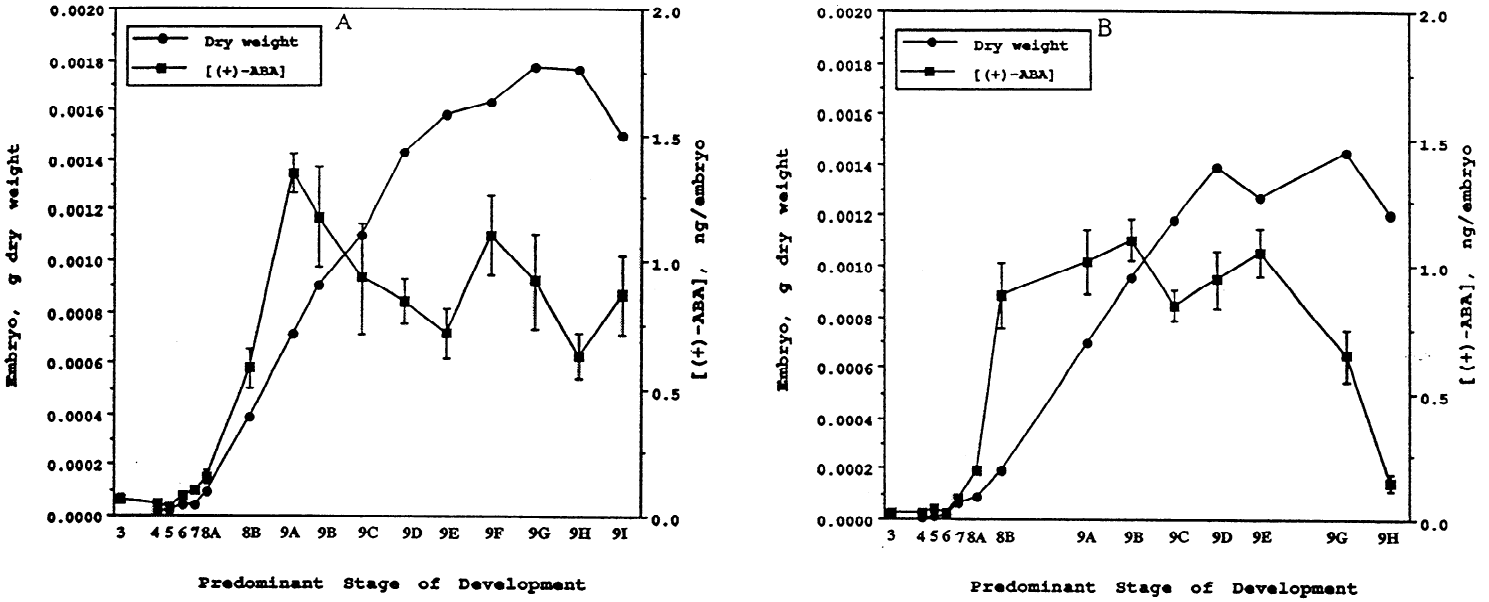

Figure 3. ABA level versus dry-weight accumulation in developing embryos from (**A**) UC and (**B**) WV from Stage 3 to cone ripening (June-October 1992). Stage 9 tissue was collected weekly and divided into additional substages A-H. Error bars represent the 95% confidence intervals.

It would appear that megagametophytic ABA in loblolly pine does not change significantly across development (Figure 1). The apparent decline in ABA depicted in Figure 2 appears primarily attributable to an increase in dry weight during development, not to a drop in ABA. Results suggest that on a per-organ basis, embryonic ABA may be imported from the megagametophyte, especially during early development when moisture levels are high and the tissues are in close contact.

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